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Atlantic Research Corporation

901 N. Columbus St.

Alexandria, Virginia

Subject: Synthesis of Rubber by Microorganisms
Status Report - 1 January to 31 March, 1956
ONR Project NR330 - 033
Contract NONR 1233(00)

Submitted to: Chief, Office of Naval Research
Navy Department
Washington 25, D. C.
Attention: Code 425

Submitted by: Atlantic Research Corporation

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Summary

Two of the mutants derived from *Lactaria subdulcis* (P) by irradiation with electrons were grown in submerged culture, using a two-week culture period, until sufficient mycelia were harvested for extraction and identification of rubber. The extractions are underway.

The clear, aqueous filtrates from these cultures, after separation of mycelia, were shaken with benzene, and the benzene extracts reduced under nitrogen to small volume. The aqueous phase yielded 0.67% (based on dry weight of mycelia) of a "sticky", "leggy", rubbery polymer. The polymer was insoluble in water, alcohol, and acetone, and was not hydrolyzed by hot alkali. The material was submitted to Dr. McLaughlin of the Walter Reed Hospital for infra-red analysis. This is the first evidence of extra-cellular production of rubber by species of *Lactaria*. Lysis of cells as the source of polymer is considered improbable since the culture period was short and the quantity of elastomer relative to the total expected from the mycelia was large. Filtrates from the culture of other species of *Lactaria* when extracted with benzene have yielded no "rubbery" polymer.

Lactaria mucida, which grows slowly at temperatures above 23°C, is being cultivated on the shaker. Production of sufficient mycelia for extraction and subsequent examination for the presence or absence of rubber is expected by the middle of April.

Experiments designed to determine the feasibility of direct extraction of mycelia with benzene are in progress. Mycelia from still cultures of *L. subdulcis* (P) were used for this study.

Pure cultures of the following species of *Lactaria* were sent to Dr. Leonard for the type culture collection and to Dr. Tsuchiya of the Northern Regional Laboratories (USDA): *L. paludinella*, *L. piperata*, *L. subdulcis*, *L. subdulcis* (P), *L. torminosa*. Pure cultures of other species of interest, including the irradiated mutants, will be mailed in April.

Since cyclitols are the predominant carbohydrates of *Hevea* and *Lactaria* the species of *Lactaria* of our collection were characterized on media based on mannitol. Mannitol, which occurs in the sporophores of *Lactaria*, was substituted for dextrose in the Sabouraud medium.

Experimental

Cultivation, Separation, and Extraction of Mycelia of the Mutants Obtained from L. Subdulcis (P) by Irradiation with Electrons

The mutants obtained from *L. subdulcis* (P) by irradiation with electrons, i.e., *L. subdulcis* (P- $\gamma\beta$) induced by direct irradiation under the 2,000,000 volt beam from a Van de Graaff Electrostatic generator at a dosage of 1×10^6 rep, and *L. subdulcis* (P- γ) induced by indirect irradiation at the same dosage, were cultivated on the shaker to get material for extraction and the determination of their ability to produce rubber on liquid, synthetic media. Resolution of the feasibility of this approach for the production of high-yielding, rubber-producing strains of fungi before terminating the research program was deemed important.

Erlenmeyer flasks containing 100 ml. of an aqueous medium consisting of 2.5% Fleischmann's Diamalt syrup, 0.5% malt extract and 0.5% neopeptone were inoculated with the mutants, using 5% inoculum. The pH of the medium after autoclaving was 5.0. Twenty flasks of each mutant were placed on the shaker and cultivated for a period of two weeks at temperatures ranging from 22 - 25°C. The cultures were then removed, the mycelia separated from the media by filtration, quick-frozen at -18°C, and preserved in three volumes of hot ethanol containing hydroquinone (.040 gms/liter) as antioxidant. This process was repeated until approximately 80 grams (dry wt. basis-calc.) of mycelia of each mutant were harvested. Sixteen liters of culture medium, for each mutant, were required.

The clear filtrates from the cultures of the mutants, after separation of mycelia, were combined and extracted with benzene. Five hundred milliliter aliquots of the filtrates were shaken first with 100 milliliters of benzene in a separatory funnel, and then with 75 milliliters of solvent. The benzene extracts from 28 liters of medium were combined and reduced to a volume of 100 milliliters. Solvent was removed with a stream of oxygen-free nitrogen. Phenyl- β -Naphthylamine, 0.1% was added as antioxidant. An aliquot showed the benzene solubles to be 0.947 gms or 0.68% on the mycelia (138 grams-dry wt.) produced by the media. The residue from the aliquot was a sticky, "leggy", rubbery polymer. It was not soluble in water, alcohol, or acetone. The remaining benzene solution blanketed with nitrogen was submitted to Dr. McLaughlin of Walter Reed Hospital for infra-red analysis.

Response Variation of Species of Lactaria
on Media with Mannitol as the Carbohydrate

Cyclitols, quebrachitol (or inositol) in Hevea and mannitol in Lactaria, are the predominant carbohydrates of tissues of these rubber-bearing plants. These carbohydrates may have a role in rubber synthesis. The species of Lactaria, including the mutants, were characterized as to response on the following media:

(a) Mannitol agar

Neopeptone	5	gm
d-Mannitol	20	gm
Bacto agar	7.5	gm
Deionized water	500	ml
pH after sterilization	6+	

Note: This is the Sabauraud medium with mannitol substituted for dextrose.

(b) Mannitol-Diamalt Agar

Neopeptone	2.5	gm
Fleischmann's Diamalt	7.5	gm
d-Mannitol	7.5	gm
Bacto-agar	7.5	gm
Deionized water	500	ml
pH after sterilization	5+	

With Fleischmann's Diamalt syrup as a source of accessory food factors and minerals the Lactaria are readily adapted for growth on media containing mannitol. Growth on the mannitol agar did not differ significantly from that on Sabauraud-dextrose. The response of the various species is shown in Tables VIIB, VIIIB, XVIII, XVIIIIB, XVIIIIC, XIX, XIXB.

Cultivation of Lactaria Mucida

Colonies of L. mucida grown on media containing mannitol are very rubbery in texture. Exudates from the colonies when rubbed to dryness between the fingers are "sticky and leggy". This fungus was grown in shaker culture in the following aqueous mediums:

Fleischmann's Diamalt syrup	0.5%
Mannitol	1.5%
Neopeptone	0.5%

Inoculum was 5% and period of growth at 22 - 25°C was one month. Some difficulty has been experienced with the cultivation of this species,--the cultures undergo lysis within a period of forty-eight hours when the temperature exceeds 70°F. Forty grams (dry weight) have been harvested. Sufficient material for extraction and characterization of the rubber is expected by the middle of April.

"Vistex" Extractions

Mutants (irradiated) from *L. subdulcis* (P) were grown in still culture in Fernbach flasks, using 200 mls per flask of medium consisting of 2.5% Fleischmann's Diamalt, 1.5% mannitol and 0.5% neopeptone. The mycelia were harvested, quick-frozen at -18°C , and thawed in hot ethanol. The mycelia were separated from the ethanol and extracted with benzene. Results will be presented in the final report.

Perpetuation of Cultures

Pure cultures of the following species of *Lactaria*, with instructions for culture were prepared and mailed to Dr. Leonard of NRL and to Dr. Tsuchiya of the Peoria Regional Laboratory (USDA):

- L. paludinella*
- L. piperata*
- L. subdulcis* (Black parent strain)
- L. subdulcis* (Spontaneous pink mutant)
- L. torminosa*

Pure cultures of other species will be mailed upon termination of the project in April.

Trace Element Diamalt

TABLE VII B

Response Variation of Irradiation-Induced Mutants

9 days		Giant Colonies				30 days	
Species	Diameter	Pigmentation			Reverse	Texture-Remarks	Compared to Norm
		Surface	Margin	Top			
Gautiera γ	85 mm	Patches, spiky submerged	Fringed	Whitish patches	None	Coarse stretchy membrane, many clumps of crystals, vinegar odor	More crystals
groveiciens $\gamma\beta$	85 mm	Thread like patches, wrinkled	Fringed	Whitish patches	None	Coarse, tacky membrane, more crystals, vinegar odor	Many more crystals more tacky
Lactaria murida γ	17 mm	Highly raised woolly	Undulate	Whitish	Brown	Chitinous, good growth	More pigment
Lactaria γ	20 mm	Spiky center glabrous	Channeled	Light pink	Light red	Thick skin like, pigment faded	Similar
subdulcis P $\gamma\beta$	20 mm	Tufted center channeled	Undulate	Pink	Orange	Thick skin like, pigment faded	Similar
Lactaria γ	50 mm	Highly raised silky	Undulate	White, watery exudate	Ochre	Stretchy, makes legs, good growth	More rubbery, some crystals
torminosa $\gamma\beta$	45 mm	Raised, silky	Undulate halo	White, watery exudate	Pale ochre	Skin like, scapy, growth submerged, pigment faded	Less rubbery, many large crystals
Lactaria γ	50 mm	Velvety channeled	Hyaline halo	Rose beige White rays, border	Pale ochre	Skin like, not tacky	Less pigment
vellerea $\gamma\beta$	52 mm	Velvety	Halo	Whitish, pink ring	Pale ochre	Skin like, not tacky	Very little pigment

Casamino Acid - Diamalt

TABLE VIII B

Response Variation of Irradiation-Induced Mutants

Giant Colonies 30 days							
Species	Diameter	Surface	Margin	Pigmentation		Texture-Remarks	Compared to Norm
				Top	Reverse		
Gautiera	85 mm	Submerged silky patches	Fringed	Whitish on hyaline	None	Tacky, makes legs, yeasty odor	Similar
groveolens $\gamma\beta$	85 mm	submerged, fine silky patches	Fringed	Whitish on hyaline	None	Tacky, makes legs, vinegar odor	Finer growth, odor of acetic acid
Lactaria mucida	16 mm	Highly raised channeled	Undulate	Amber to hyaline	Brown	Rubbery, makes legs, mushroom odor	Larger, more pigment
Lactaria	20 mm	Spiky center channeled	Undulate	Dark orchid	Blood red	Stretchy, skinlike, pruinose center	Similar
subulcis P $\gamma\beta$	20 mm	Spiky center channeled	Undulate	Vinaceous	Dark red	Thin skinlike, tacky large pruinose center	More pigment, more tacky
Lactaria	55 mm	Raised cottony	Entire	White	Pale ochre	Good growth, tacky skinlike	More tacky
torminosa $\gamma\beta$	57 mm	Raised, cottony hyaline border	Entire	White and hyaline	None	Tough, skinlike, tacky submerged sectors	More submerged growth rubbery
Lactaria	50 mm	Velvety, flat	Entire wide halo	Deep and light pink rays	None	Skinlike, tacky	White sectors
vellerea $\gamma\beta$	50 mm	Velvety, flat	Entire	Very pale pink, rings	None	Tough, skinlike, tacky	Very little pigment

TABLE XVIII

Response-Variation

9 days							30 days
Species	Diameter	Surface	Margin	Top	Pigmentation	Texture-Remarks	
<i>Gautieria groveolens</i>	85 mm	Raised, hairy	Dendritic	White	Yellowish	Very stretchy membrane, submerged, odor of vinegar	
<i>Bulgaria rufa</i>	50 mm	Raised, coarse hairy	Fringed	White	Light tan	Heavy abundant growth, spots of brown pigment, no odor	
<i>Lactaria chrysorhea</i>	25 mm	Coarse spiky center	Undulate	Grey brown center, white		One plate shows possible coral pink mutant, good growth, chitinous, musty odor	
<i>Lactaria paludinella</i>	53 mm	Very highly raised, cottony	Slightly irregular	White	Light ochre	Good growth, reverse ochre leathery	
<i>Lactaria piperata</i>	30 mm	Highly raised silky	Wide hyaline border	White	Orange	Good growth, wide hyaline halo stretchy, skin-like mat	
<i>Lactaria mucida</i>	12 mm	Very highly raised, cottony	Channeled	Off white	Brown	Very good growth, larger mat on this medium, makes legs, mushroom odor	
<i>Lactaria subulicis</i> (B)	20 mm	Flat, smooth	Undulate	Chocolate, white border	Brown	Whitish pruinose off, wide halo tacky	
<i>Lactaria subulicis</i> (P)	22 mm	Spiky center glabrous	Channeled	Light pink, dark pink ring	Rose red pink diffuses	Good growth, pigment fades tacky	
<i>Lactaria forminosa</i>	55 mm	Highly raised cottony	Entire	White	None	Good growth, reverse ochre, cottony, leathery	
<i>Lactaria vellerea</i>	57 mm	Flat velvety	Undulate	Pink, darker rings	White	Good growth, stretchy mat, tacky	

Response Variation of Irradiation-Induced Mutants

Species	Diameter	9 days			Giant Colonies			30 days		Compared to Norm
		Surface	Margin	Top	Pigmentation	Reversal	Texture-Remarks			
Gautieria	Y	Raised, silky	Somewhat dendritic	White	White	Yellowish	Stretchy membrane, tacky, submerged, vinegary			Less tough membrane
grovecleus $\gamma\beta$	85 mm	Raised, fine silky	Less dendritic	White	White	Yellowish	Membrane tears rather than stretches			Very delicate membrane
Lactaria mucida	Y 11 x 20	Raised, spiky	Crenulate	Whitish and amber	Brown		Good growth, makes legs mushroom odor			Better growth, less exudate
Lactaria	Y 19 mm	Glabrous slightly channeled	Undulate	Lavender		Pink, some diffuse pink	Good growth, red reverse tacky			Better growth, more pigment, pruinose
subdulcis P $\gamma\beta$	25 mm	Glabrous channeled	Undulate	Rose		Red-diffuse light red	Very good growth, very tacky			Better growth, much more pigment
Lactaria	Y 60 mm	Highly raised cottony	Entire	White		None	Good growth, reverse ochre, leathery, tacky			More pigment, more tacky
terminosa $\gamma\beta$	60 mm	Raised cottony	Entire, hyaline border	White		None	Good growth, reverse light orange, stretchy, tacky			More tacky
Lactaria	Y 54 mm	Flat, velvety	Entire	Pink rings white sectors		White	Good growth, leathery no odor			Less tacky, white sectors
vellerea $\gamma\beta$	47 mm	Flat, velvety channeled	Undulate	Whitish, pale pink rings		Yellowish	Good growth, pale, tacky, perfume-odor			Less pigment, more tacky

Response Variation of Irradiation-Induced Mutants

9 days		Giant Colonies					30 days	
Species	Diameter	Surface	Margin	Top	Pigmentation	Reverse	Texture-Remarks	Compared to Norm
<i>Lactaria chrysochea</i>	22 mm	Coarse hairy	Slightly undulate	Grey center wide white border		None	Very good growth parchment-like	Larger mat, more grey aerial mycelium
<i>Lactaria</i>	55 mm	Highly raised woolly	Entire	Off white minute droplets		None	Skin-like, flat, pale buff stretchy mat	More pigment in mycelium, less pigment reverse
<i>paludinella</i> $\gamma\beta$	55 mm	Highly raised fine densely packed	Entire	White		Light brown	Skin-like, cottony, white	White mycelium, more pigment reverse
<i>Lactaria piperata</i>	30 mm	Highly raised silky	Entire, narrow hyaline border	White		Deep orange-yellow	Good growth, brownish pigment, leathery	Narrow hyaline halo less pigment
<i>Lactaria</i>	25 mm	Raised, rough	Undulate	Brown, narrow white border		None	Good growth, not pruinose rugose, delicate, tacky	More rugose, darker pigment
<i>subulcis</i> B $\gamma\beta$	20 mm	Raised, rough	Undulate	Brown, fine white border		None	Fair growth, rugose, not pruinose, not tacky	Smaller mat, darkest pigment

TABLE XIX

Response-Variation

Species	Diameter	Surface	Margin	Giant Colonies			Texture-Remarks
				Top	Pigmentation	Reverse	
Lactaria mucida	10 x 15 mm	Raised, cottony	Cottony	White	None	None	Leathery, becoming pigmented, magentared after exposure to light, poor growth
Lactaria paludinella	20 x 23	Raised furry	Cottony	White	Pale orange		Tacky, good growth
Lactaria subdulcis B	17 x 10	Flat smooth	Undulate	Brown center off white	Light brown		Not tacky, very poor growth
Lactaria subdulcis P	6 x 18	Flat smooth	Entire	Whitish	Yellow		Leathery, poor growth yellow pigment diffuses in medium
Lactaria torminosa	50 x 52	Raised, dense silky	Entire	White	Light brown		Leathery, good growth, brown rings, reverse
Lactaria vellerea	30 x 42	Flat, velvety	Irregularly lobed	Rose beige center, white	None		Tacky, wide halo, becoming lavender irregularly reniform

TABLE XIX B

Response Variation of Irradiation-Induced Mutants

Species	Diameter	Surface	Margin	Pigmentation		Giant Colonies		Compared to Norm
				Top	Reverse	30 days	Texture-Remarks	
Lactaria mucida	Y 10 x 13	Raised spiky	Spiky	White	None	Not rubbery, good growth, much violet to magenta pigment	Mature colony "are" more pigment developed	
Lactaria	Y 11 x 15	Flat	Entire	White	None	Skin like, stretchy, good growth, pink, orange reverse	Better growth, strong diffuses into medium	
subulvis Pyl	12 x 16	Flat	Entire	White	None	Stretchy, pale pink, orange reverse	Much better growth, strong diffuses into medium	
Lactaria	Y 47 x 50	Raised, dense silky	Entire	White	Light brown	Leathery, stretchy, good growth, reverse brown rings	More stretchy	
terminosa Y	50 x 57	Raised, dense silky	Entire	White	Pale tan	Tacky, good growth, no rings reverse	More elastic	
Lactaria	Y 30 x 35	Flat velvety	Irregularly lobed	Whitish lavender sector	None	Tacky, whitish pink lavender sectors	Less pigment, irregularly reniform	
vellerea Y	22 x 35	Flat velvety	Irregularly lobed	Hyaline whitish center	None	Tacky, white, mostly submerged growth	Much less pigment, irregularly reniform	

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